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# T Cells Reactive to an Inducible Heat Shock Protein Induce Disease in Toxin-induced Interstitial Nephritis

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## Summary

T cells reactive against immunodominant regions of inducible heat shock proteins (HSPs) have been identified in the chronic inflammatory lesions of several experimental autoimmune diseases. Since HSPs are known to be induced by a number of renal tubular epithelial cell toxins associated with chronic interstitial nephritis, we investigated the relevance of HSP expression and T cell reactivity to HSP70 in a model of progressive inflammatory interstitial nephritis. Chronic administration of cadmium chloride ( $\text{CdCl}_2$ ) to SJL/J mice induces HSP70 expression in renal tubular cells 4–5 wk before the development of interstitial mononuclear cell infiltrates.  $\text{CdCl}_2$  also induces HSP70 expression in cultured tubular epithelial cells from SJL/J mice.  $\text{CD4}^+$ ,  $\text{TCR-}\alpha/\beta^+$  T cell lines specific for an immunodominant HSP peptide are cytotoxic to heat stressed or  $\text{CdCl}_2$ -treated renal tubular cells. Such HSP-reactive T cells mediate an inflammatory interstitial nephritis after adoptive transfer to  $\text{CdCl}_2$ -treated mice at a time when immunoreactive HSP70 is detectable in the kidneys, but before the development of interstitial mononuclear cell infiltrates. T cells isolated from the nephritic kidneys of mice treated with  $\text{CdCl}_2$  for 13 wk are also cytotoxic to heat shocked or cadmium-treated tubular cells. These kidney-derived T cells additionally induced interstitial nephritis after passive transfer, indicating their pathogenic significance. Our studies strongly support a role for HSP-reactive T cells in  $\text{CdCl}_2$ -induced interstitial nephritis and suggest that the induction of HSPs in the kidney by a multitude of “non-immune” events may initiate or facilitate inflammatory damage by HSP-reactive lymphocytes.

Chronic interstitial nephritis is an important cause of chronic renal failure and is epidemiologically associated with many diverse etiologies including excessive exposure to heavy metals, certain drugs, and ischemia. Despite such diverse etiologies, the pathologic lesion is remarkably similar in progressive interstitial disease consisting of a mononuclear cell infiltrate, tubular atrophy and dilatation, and interstitial fibrosis (1). While it is clear that such pathologic abnormalities can be initiated as the result of autoimmune T and B cell responses to renal antigens (2), many forms of chronic interstitial nephritis have been historically classified as toxic nephropathies with functional and structural abnormalities attributable to nonimmune damage to renal parenchymal cells.

The role of heat shock proteins (HSPs)<sup>1</sup> in the development of autoimmune disease has been the focus of recent in-

terest (3). HSPs are a highly conserved family of proteins, induced by many cellular stresses including heat shock, infection, ionizing radiation, antibiotics, and heavy metals. They perform important intracellular functions including protein trafficking, oligomer assembly, binding to damaged or aberrant proteins, and prevention of toxic aggregate formation (4–8). Members of the 70-kD family of HSPs (HSP70), which include microbial HSP65, are highly immunogenic.  $\text{TCR-}\alpha/\beta^-$  and  $-\gamma/\delta^-$  expressing T cells specific for mycobacterial HSP65, a constituent of purified protein derivative, can be isolated from human, rat, and murine hosts (9, 10). These T cells, initially activated by microbial HSPs, crossreact with endogenous HSPs (11). HSP-reactive lymphocytes play a role in the pathogenesis of several experimental autoimmune diseases, including adjuvant arthritis (12), autoimmune insulinitis and diabetes (13), and encephalomyelitis (14). In these experimental models, HSP-reactive lymphocytes are found in pathological lesions along with T cells reactive to organ-specific antigens, such as cartilage proteoglycans, islet cell antigens, and myelin basic protein.

<sup>1</sup> Abbreviations used in this paper: DTH, delayed-type hypersensitivity; HEL, hen egg lysosome; HSP, heat shock protein; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

To investigate the hypothesis that HSP70-reactive T cells may result in renal injury in the setting of toxin-induced HSP expression, we used a model of chronic CdCl<sub>2</sub> administration to SJL/J mice. We examined HSP70 expression in CdCl<sub>2</sub>-treated renal tubular cells and in the kidneys of mice given CdCl<sub>2</sub>. HSP70-specific CD4<sup>+</sup>, TCR- $\alpha/\beta$ <sup>+</sup> T cell lines were isolated from SJL mice, either after immunization with synthetic peptides derived from HSP65 or directly from the nephritic kidneys of mice chronically treated with CdCl<sub>2</sub>. We examined the reactivity of these T cells against endogenous tubular cell HSP70, both with cultured cells and in the whole animal model. Our results suggest that HSP-reactive T cells play a role in the pathogenesis of chronic toxin-induced interstitial nephritis.

## Materials and Methods

**Mice.** SJL/J mice (H-2<sup>d</sup>) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a University of Pennsylvania animal facility.

**Kidney Cell Lines.** MCT cells, a proximal tubular epithelial cell line derived from SJL/J mice (15), were grown as previously described.

**T Cell Lines.** The HSP-1 cell line was established as follows. SJL mice 10–12 wk of age were immunized in their footpads and axilla with a peptide corresponding to amino acids 180–196 of *Mycobacterium bovis* HSP65, with the amino acid sequence TFG-LQLELTEGMRFD (synthesized by the Protein Chemistry Laboratory of the University of Pennsylvania), 3 mg/ml in incomplete Freund's adjuvant (IFA). Draining lymph nodes were harvested 2 wk after immunization, minced, and pressed through an 80-gauge sieve. The resulting cell suspension was washed twice with T cell media (RPMI 1640 with 10% FCS, 5% NCTC 109 (GIBCO BRL, Gaithersburg, MD), and  $2 \times 10^{-5}$  M 2-ME) and plated at  $2 \times 10^6$  cells/well in T cell media with 2  $\mu$ g/ml HSP 180–196 and  $10^6$  irradiated syngeneic splenocytes. After three passages with HSP 180–196 and irradiated splenocytes, Lympholyte (Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada) purified T cells were subsequently maintained in culture as above with the addition of 20% MLA-144 cell supernatant as a source of IL-2 and other growth factors (16). The HEL cell line was derived from SJL/J mice immunized with 3 mg/ml hen egg lysozyme (HEL; Sigma Chemical Co., St. Louis, MO) in CFA and boosted with two injections of HEL/CFA 7 and 14 d after initial immunization. Draining lymph nodes were harvested 21 d after initial immunization, and T cells were separated and grown in long-term culture as described above using 2  $\mu$ g/ml HEL as antigen. To derive the CdCl<sub>2</sub> cells, nephritic kidneys of SJL/J mice after 13 wk of treatment with 1.25 mg/kg/d CdCl<sub>2</sub> were minced, gently pressed through an 80 gauge screen, washed four times with T cell media, and gently pressed through a 140-gauge screen to separate glomeruli from tubular fractions. The tubular-enriched suspension was washed twice with RPMI, and plated with  $10^6$  syngeneic irradiated splenocytes/well in T cell media with HSP 180–196 at 2  $\mu$ g/ml, and subsequently maintained in long-term culture under the same conditions as the HSP-1 T cell line.

**Delayed Type Hypersensitivity (DTH) Testing.** SJL/J mice were pretreated with 20 mg/kg cyclophosphamide i.p. 24 h before testing (17). 8 h before receiving T cells, pretreated mice were injected in their footpads with 25  $\mu$ l PBS (right side) or antigen (left side) (18). T cells at 8–10 d post passage were purified by centrifugation

through Lympholyte, washed twice in PBS, and injected into the tail veins of the pretreated mice ( $40\text{--}45 \times 10^6$  cells/mouse). Footpad thickness as an assessment of DTH response was measured with a spring loaded micrometer (Mitutoyo model 7308; Schlesinger's for Tools, Tarrytown, NY) by an observer blinded to the experimental design. DTH response was expressed as the mean difference between the antigen-challenged footpad and the PBS-injected footpad in inches  $\times 10^{-3} \pm$  SEM.

**Western Blotting Studies.** Confluent MCT cells (48 h post passage) cultured in DMEM with 10% FCS were treated with heat shock (41°C for 2 h), or with CdCl<sub>2</sub> ( $3.4 \times 10^{-6}$  M for 4, 12, or 16 h), or left untreated. Total cellular lysates were prepared (19) and protein content of the lysates was measured (20). 50  $\mu$ g of each lysate was resolved by SDS-PAGE, transferred to nitrocellulose, and probed using a 1:2,000 dilution of a murine monoclonal anti-HSP 70 antibody (RNP1197; Amersham Corp., Arlington Heights, IL) in TBS/0.1% Tween 20, followed by horseradish peroxidase-labeled goat anti-mouse IgG (Boehringer Mannheim Corp., Indianapolis, IN) diluted 1:3,000 in TBS/0.1% Tween 20. The blots were developed with the ECL Western blotting detecting system (Amersham Corp.) according to the manufacturer's instructions.

**Immunohistochemistry.** HSP expression within the kidney was determined during the course of interstitial nephritis. For this purpose, whole kidneys were harvested from cadmium-treated and control animals at 3, 6, and 10 wk, formalin fixed, sectioned, deparaffinized, and evaluated for HSP expression with the anti-HSP70 antibody (RPN1197; 1:1,000 in PBS/0.1% BSA), and the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) according to manufacturer's instructions.

**Cytotoxicity Assays.** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay of cell viability (21, 22) has previously been adapted by our laboratory for measuring T cell-mediated cytotoxicity to tubular epithelial cells (23). Single cell suspensions of MCT cells (target cells) were plated in 96-well flat bottom plates at  $3 \times 10^4$  cells/well in DMEM with 10% FCS and 0.5  $\mu$ g/ml mitomycin C and incubated at 37°C, 5% CO<sub>2</sub> for 6 h to permit attachment to the bottom of wells. After attachment, cells were either left unmanipulated, treated with heat shock (41°C  $\times$  2 h), or incubated with CdCl<sub>2</sub> ( $3.4 \times 10^{-6}$  M) for 4, 12, or 16 h. All wells were gently washed and replated with T cell media containing 0.5  $\mu$ g/ml mitomycin C. T cells at end passage were separated from splenocytes by Lympholyte centrifugation, washed, resuspended in T cell media with 0.5  $\mu$ g/ml mitomycin C and added to the wells containing MCT cells at effector/target ratios of 5:1, 10:1, 25:1, and 50:1, with a final volume of 200  $\mu$ l/well in all wells. Identically treated triplicate wells of MCT cells that were not incubated with T cells served as controls for each plate. After a 20-h incubation at 37°C, the wells were gently washed with warmed media to remove nonadherent cells, and replated with DMEM/10% FCS and 0.5  $\mu$ g/ml mitomycin C, 180  $\mu$ l/well and 5  $\mu$ g/ml MTT, 20  $\mu$ l/well. Plates were incubated for 4 h at 37°C to allow for dye metabolism, then the wells were aspirated and replated with 0.04 N HCl in isopropanol, 180  $\mu$ l/well and 3% SDS, 20  $\mu$ l/well. After reincubation for 1 h at 37°C, OD at 590 nm was determined (microplate kinetic reader; Molecular Devices, Menlo Park, CA). The mean and SEs of readings from each group of triplicate wells were calculated, and the percent cytotoxicity was determined by the following formula: percent cytotoxicity =  $(1 - \text{OD experimental/OD control}) \times 100$ .

**Adoptive Transfer Experiments.** Age and sex matched SJL/J mice at 6–10 wk of age were injected intraperitoneally with either CdCl<sub>2</sub> in PBS (pH corrected with NaOH to 7.4) 1.25 mg/kg/d or an equal volume of PBS alone for 6 wk. T cells at end passage

were purified by Lympholyte centrifugation, washed twice with sterile PBS, and resuspended in PBS at  $2.5 \times 10^8$  cells/ml. After anesthesia with ether, the kidney was exposed through flank incisions with the lower pole gently stabilized by traction on the perinephric fat. Experimental animals were injected with either 75  $\mu$ l of T cell suspension or PBS under the renal capsule with the aid of a dissecting microscope, after which the incisions were closed with staples (23). Animals were sacrificed one week following subcapsular transfers. Both kidneys were harvested (the contralateral side as a control), sectioned longitudinally into thirds, and fixed in 10% buffered formalin. 4-micrometer sections of paraffin embedded tissue were stained with hematoxylin and eosin and examined for cellular infiltrates and tubular atrophy by a pathologist (JET) blinded to the experimental groups. Grading scales were modified from a system used previously (23). The area of the mononuclear cell infiltrate was measured in two dimensions. The density of the infiltrate was graded on a scale of 0–4 where 0 = no inflammatory cells found in the area of abnormal appearing tubular cells; 1 = 1–10% of cell nuclei in the histologically abnormal areas were mononuclear cells; 2 = 11–25% inflammatory cells in histologically abnormal areas; 3 = 26–50% inflammatory cells in the histologically abnormal areas; and 4 = 51% or greater inflammatory cells in histologically abnormal areas. Parameters of tubular cell damage noted on histological sections included marked basophilia of tubular epithelium, increased nuclear/cytoplasmic ratio, microvacuolar cytoplasmic changes, and decreased cytoplasmic volume. Tubular cell damage in the area of the infiltrate was also graded on a scale of 0–4 with 0 = no tubular cells in the area of infiltrate demonstrating evidence of damage; 1 = 1–10%; 2 = 11–25%; 3 = 26–50%; 4 = 51% or greater of tubular cells in the area of infiltrate showing evidence of tubular damage.

**Statistical Analysis.** Data was analyzed for statistical significance at the  $p = 0.001$  level by nonparametric methods including the Kruskal-Wallis one-way analysis of variance by rank and the Mann-Whitney U test. MTT cytotoxicity assays were analysed by either the preceding methods or by analysis of variance and two-tailed T test.

## Results

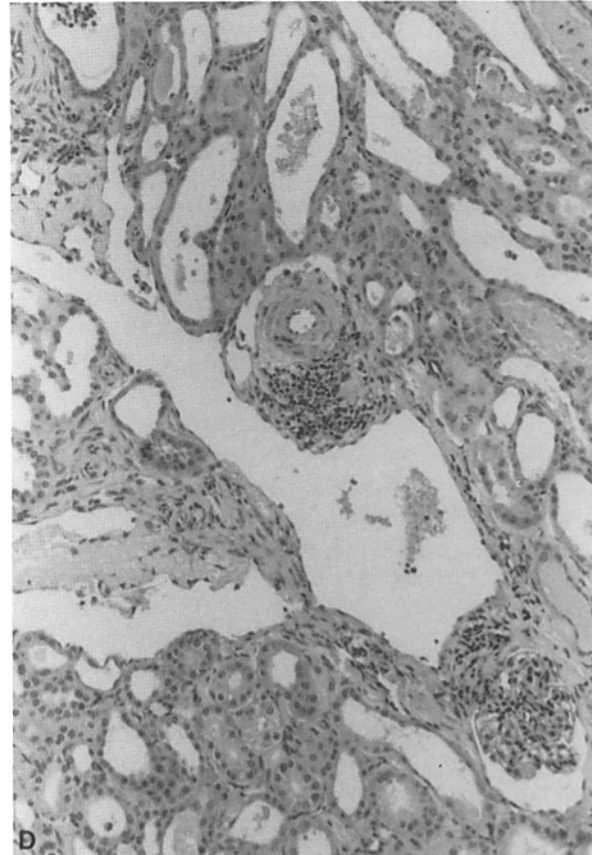
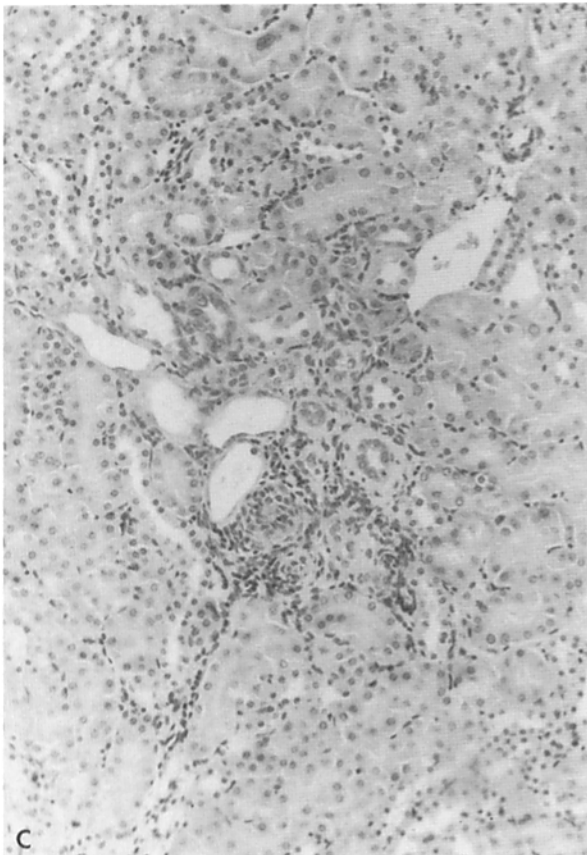
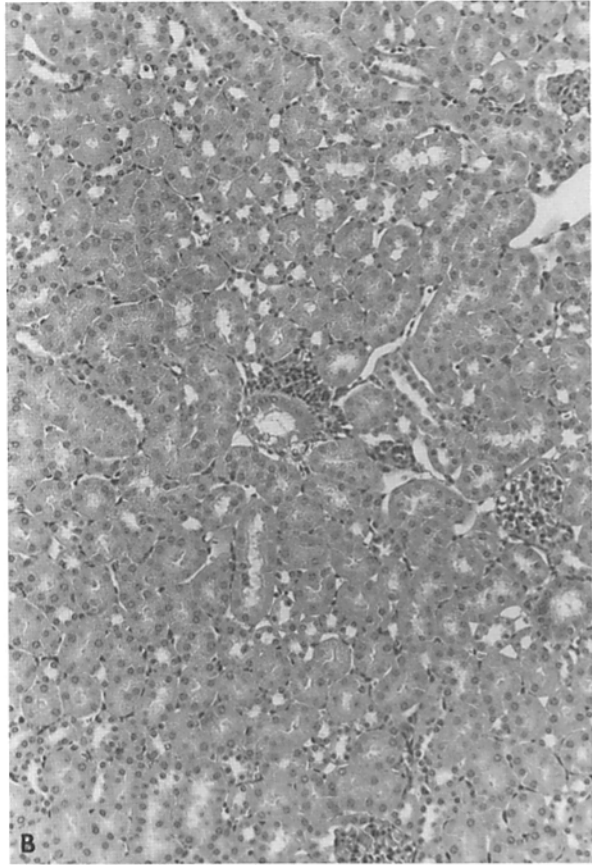
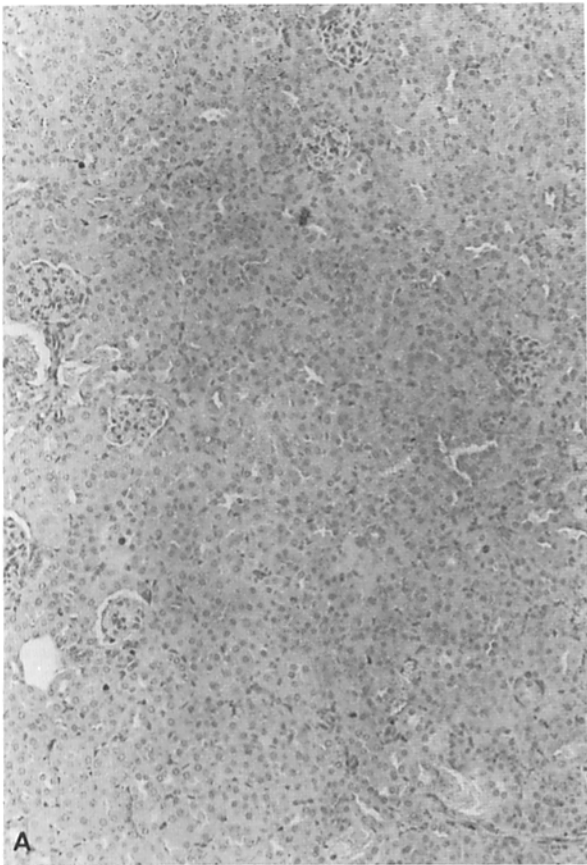
**SJL Mice Treated with CdCl<sub>2</sub> Express HSP70 in Their Kidneys before the Development of Interstitial Nephritis.** Rats chronically administered CdCl<sub>2</sub> demonstrate tubular epithelial swelling and patchy necrosis at 4–6 wk, with early interstitial infiltrates seen at 8 wk (24). We first examined the time course of disease development for CdCl<sub>2</sub>-induced interstitial nephritis in SJL/J mice. Several cohorts of SJL/J mice received CdCl<sub>2</sub> in doses ranging from 1 to 3 mg/kg/d. Their kidneys were examined at 2-wk intervals for interstitial lesions. No interstitial infiltrates were observed before 8 wk. After 6 wk of CdCl<sub>2</sub> treatment, we began to observe histologic evidence of tubular cell toxicity including loss of tubular cell eosinophilia, increased nuclear/cytoplasmic ratio, and occasional nuclear fragmentation. Focal peritubular infiltrates begin to appear at 8–10 wk (Fig. 1 b). After 11–13 wk the interstitial infiltrates were well established, and tubulitis with tubular atrophy begins to appear (Fig. 1 c). By 19–21 wk fewer mononuclear cells were evident in the interstitium, and tubular atrophy and interstitial fibrosis became more pronounced (Fig. 1 d).

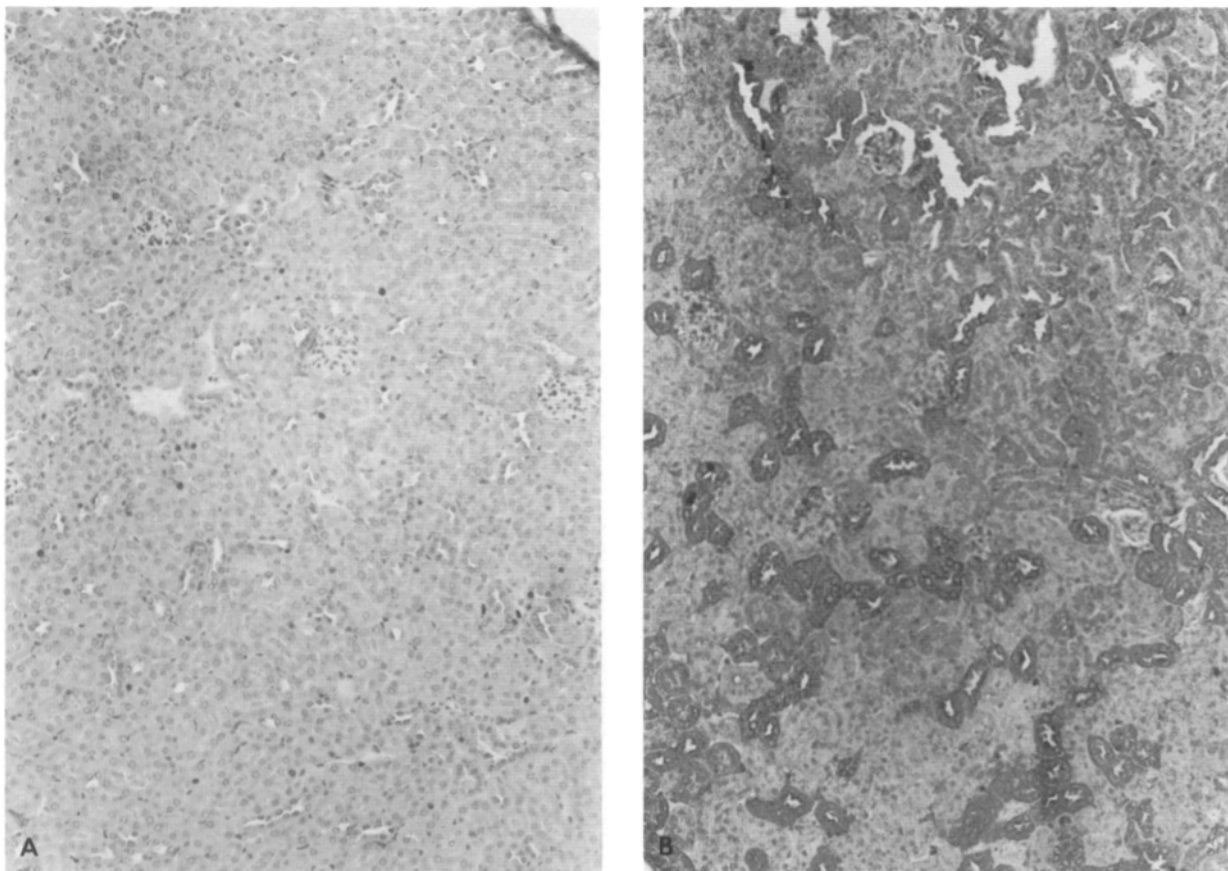
We next examined the time course of renal HSP70 expression in response to CdCl<sub>2</sub> treatment, and the relationship of HSP70 expression to development of tubular damage and cellular infiltrates. In several cohorts of SJL/J mice treated with CdCl<sub>2</sub> (doses ranging from 1 to 3 mg/kg/d) mononuclear cell infiltrates were initially observed at 8 wk of CdCl<sub>2</sub> treatment. We therefore studied HSP70 expression in mice before, at the time of, and after the appearance of interstitial mononuclear cell infiltrates. HSP70 was not detectable in untreated mice (data not shown). We first detected focal positive staining of cortical tubular epithelial cells in the inner stripe of the medulla after 3 wk of CdCl<sub>2</sub> administration (Fig. 2 B). Immunoreactive staining increased in distribution and intensity between 3 and 10 wk, when mononuclear cell infiltrates begin to appear (data not shown).

**CdCl<sub>2</sub> Induces HSP70 Expression in MCT Cells.** MCT cells are a proximal tubular epithelial cell line derived from SJL/J mice (15). We next evaluated whether this cell line also demonstrated inducible HSP70 expression. Fig. 3 demonstrates that MCT cells express HSP70 after heat shock or exposure to CdCl<sub>2</sub> for 12 or 16 h. No immunoreactive protein was detected in unmanipulated MCT cells or cells incubated with CdCl<sub>2</sub> for 4 h. The intensity of the 70-kD band increased from 12 to 16 h of CdCl<sub>2</sub> exposure. These Western blots of MCT cell lysates occasionally also demonstrated 32- and 90-kD bands after reaction with the anti-HSP 70 antibody, which likely represents crossreactivity of this mAb to other families of HSPs.

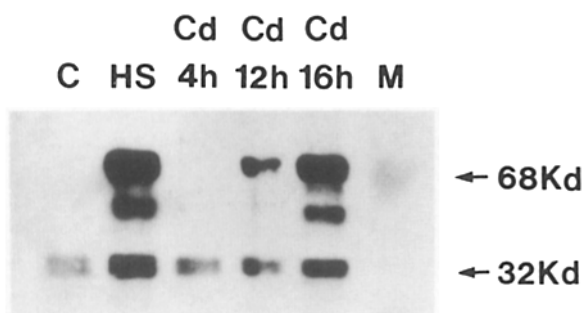
**The HSP1 T Cell Line Mediates Cytotoxicity against MCT Cells Expressing HSP70.** To investigate the potential for HSP-reactive T cells to injure renal tissue or renal cells expressing HSP70, we established a T cell line (HSP1) from the draining lymph nodes of a mouse immunized with an immunodominant peptide of HSP65. Cytofluorography studies demonstrated that HSP1 expresses TCR- $\alpha/\beta$  (not the TCR- $\gamma/\delta$  as some HSP-reactive cells do) and is CD4<sup>+</sup>, CD8<sup>−</sup> (data not shown). Fig. 4 A demonstrates that HSP1 cells mediate a significant DTH response to the HSP peptide (HSP 180-196) to which they were generated. The specificity of this response is demonstrated by the lack of a response to 3M-1(p1), a peptide sequence recognized by responding T cells in the antitubular basement membrane model of interstitial nephritis (18).

We next examined whether these HSP1 cells were cytotoxic to stressed renal tubular cells. In preliminary studies, we found that the viability of MCT cells (assessed by trypan blue exclusion) was not significantly affected by either heat shock or CdCl<sub>2</sub> treatment at the concentrations used in these experiments (data not shown). Fig. 5 A shows that HSP1 cells are cytotoxic to heat shocked MCT cells between E/T ratios of 5:1 and 50:1. This cytotoxicity showed a dose-dependent effect with increased cytotoxicity noted at the higher E/T ratios. If the MCT cells were not heat shocked, coculture with HSP1 cells never resulted in >15–18% cytotoxicity (Fig. 5 A). If instead of receiving heat shock the MCT cells were cocultured with CdCl<sub>2</sub> for graded time intervals, they also became susceptible targets for HSP1-mediated cytotoxicity (Fig. 5 B). The percent cytotoxicity at the same E/T





**Figure 2.** Immunoreactive HSP70 localized in renal tubules of cadmium-treated mice at 3, but not in PBS treated kidney tubules. Representative deparaffinized kidney sections at the indicated time points immunoperoxidase stained as described in Materials and Methods with RPN1197, antiinducible HSP70 antibody, and FITC-conjugated goat anti-mouse IgG as secondary antibody. Kidneys from PBS-treated control animals stained negatively with both antibodies. (A) Mouse kidney after 3 wk cadmium treatment, secondary antibody alone. (B) Mouse kidney after 3 wk cadmium treatment, stained with anti-HSP70 antibody.

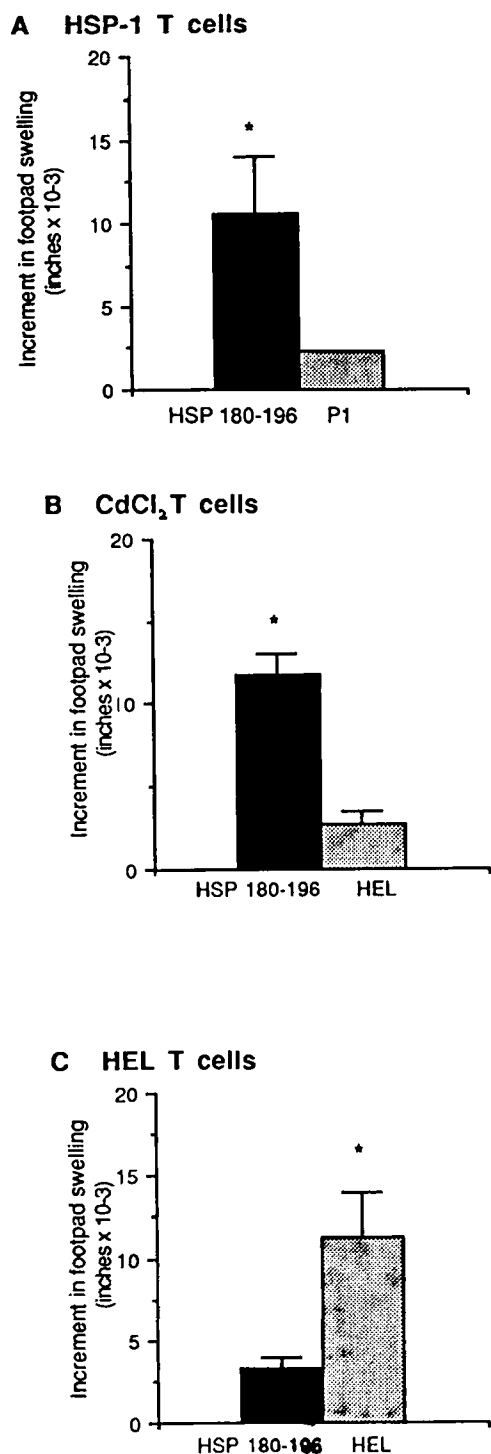


**Figure 3.** Heat shock and cadmium treatment induce expression of HSP70 in cultured proximal tubular cells. Total cellular lysates of unmanipulated control, heat-shocked, and cadmium-treated MCT cells at indicated time intervals subjected to SDS-PAGE, transferred to nitrocellulose and probed with antibody to inducible HSP70. C, control; HS, heat shocked at 41°C for 2 h; Cd 4 h, incubated with CdCl<sub>2</sub> 3.4 × 10<sup>-6</sup> M for 4 h; Cd 12 h, incubated with CdCl<sub>2</sub> 3.4 × 10<sup>-6</sup> M for 12 h; Cd 16 h, incubated with CdCl<sub>2</sub> 3.4 × 10<sup>-6</sup> M for 16 h; M, marker.

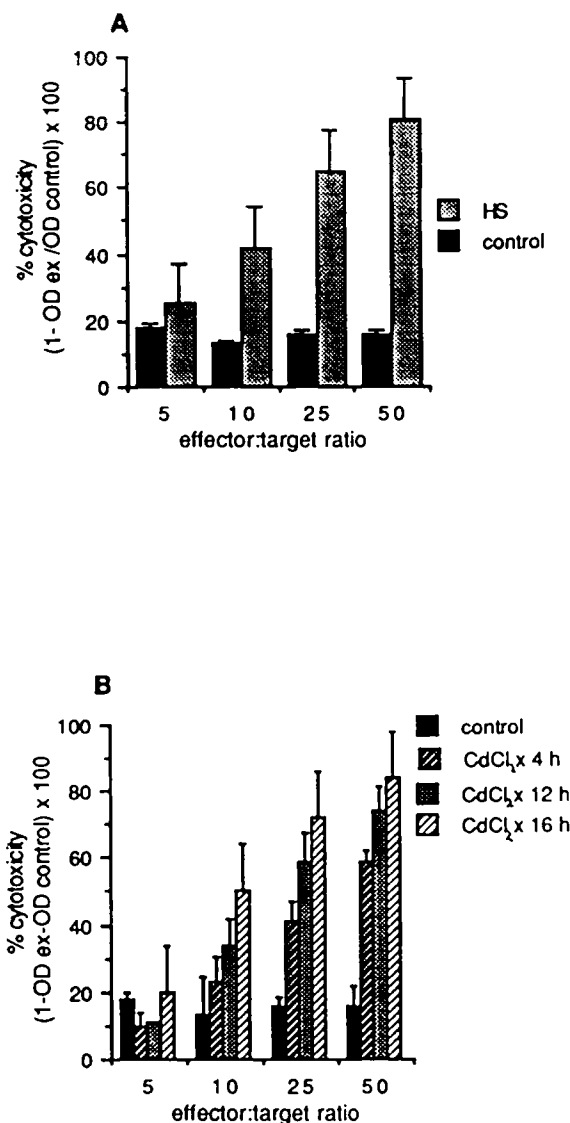
ratio was always higher in MCT cells that had been exposed to CdCl<sub>2</sub> for longer time intervals, consistent with the time course of induced HSP70 expression shown in Fig. 3. HEL-specific CD4<sup>+</sup> T cell lines do not kill either heat shocked or CdCl<sub>2</sub>-treated MCT cells (see below), indicating that this is not nonspecific cytotoxicity related to cytokines released from activated T cells.

**Subcapsular Transfer of HSP1 Induces Interstitial Infiltrates in CdCl<sub>2</sub>-treated Mice.** We next examined whether HSP1 cells could recognize their target antigen in vivo after adoptive transfer into syngeneic mice. Previous work from our laboratory has shown that the technique of subcapsular adoptive transfer can specifically differentiate nephritogenic T cells from those which do not cause disease (18, 23, 25). T cells specific for irrelevant antigens do not infiltrate the kidney after adoptive transfer. They instead remain as a transferred inoculum between the capsule and the renal parenchyma. We injected

**Figure 1.** CdCl<sub>2</sub>-induced interstitial nephritis. Hematoxylin- and eosin-stained sections of SJL mouse kidneys showing representative histologic changes at the time points indicated. (A) PBS-treated mouse kidney 15 wk; (B) focal peritubular infiltrate at 9 wk cadmium treatment; (C) interstitial infiltrates with tubulitis and tubular atrophy at 13 wk cadmium treatment; (D) advanced tubular atrophy and dilatation at 19 wk cadmium treatment.



**Figure 4.** DTH response testing demonstrates antigen specificity of T cell lines. Antigen or PBS was injected into footpads 8 h before transfer of T cells. Footpad swelling was measured 24 h after T cell transfer and expressed as difference between PBS and antigen injected footpads in inches  $\times 10^{-3} \pm$  SEM for all cell lines tested. (A) HSP-1 T cell line; (B) CdCl<sub>2</sub> T cell line; (C) HEL T cell line. Nonparametric statistical analysis using Kruskal-Wallis one-way analysis of variance by ranks and Mann-Whitney U test, \* $p < 0.001$ .



**Figure 5.** HSP-1 T cells are cytotoxic to heat-shocked and cadmium-treated tubular cells. MCT cells that were either heat-shocked, treated with CdCl<sub>2</sub> for varying intervals or unmanipulated were incubated with either HSP-1 T cells at 37°C for 20 h and percent cytotoxicity determined by metabolism of MTT as described in Materials and Methods. Each data bar represents the mean of triplicate samples from a representative experiment. A statistically significant difference between cytotoxicity directed against heat-shocked and unmanipulated cells was seen at all E/T ratios greater than 5:1. Additionally, a statistically significant difference in cytotoxicity was observed in a dose-dependent manner between all cadmium exposure times evaluated for E/T ratios greater than 5:1. (A) HSP-1 T cells with heat-shocked and control MCT cells, (B) HSP-1 T cells with cadmium-treated and control MCT cells. Nonparametric statistical analysis using Kruskal-Wallis one-way analysis of variance by ranks and Mann-Whitney U test,  $p < 0.05$ .

HSP1 cells or PBS under the renal capsules of syngeneic recipients who had either been treated with PBS or CdCl<sub>2</sub> for 5 wk. Renal tissue from these animals was histologically evaluated 1 wk after adoptive transfer of cells. Table 1 compares the maximal area of infiltrates, density of inflammatory



**Table 1.** HSP-reactive T Cells Induce Interstitial Nephritis after Subcapsular Transfer

Pretreatment/cells	Infiltrate area	Inflammatory cell density	Tubular damage
CdCl <sub>2</sub> /HSP-1 (n = 9)	8.62 mm <sup>2</sup> ± 4.01*	2.8 ± 0.3 <sup>†</sup>	3.7 ± 0.5 <sup>§</sup>
CdCl <sub>2</sub> /PBS (n = 8)	2.17 mm <sup>2</sup> ± 1.97	0.8 ± 0.3	1.25 ± 0.6
PBS/HSP-1 (n = 7)	0.26 mm <sup>2</sup> ± .09	1.3 ± 0.5	2.3 ± 0.7
PBS/PBS (n = 7)	0.006 mm <sup>2</sup> ± .006	0.3 ± 0.3	0.6 ± 0.6

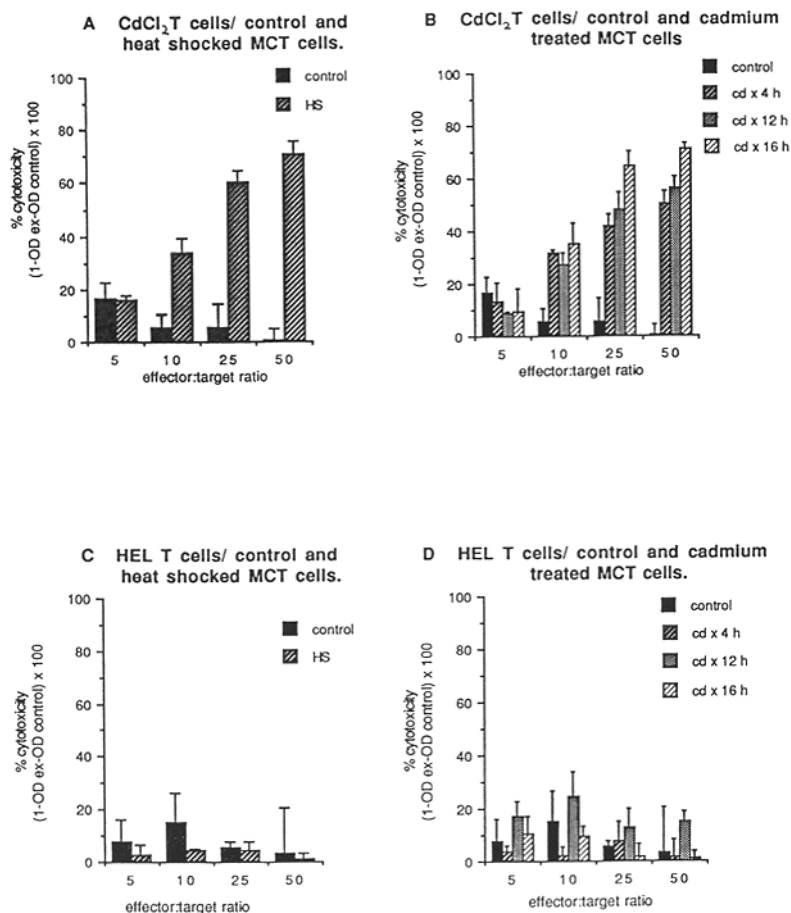
Animals were injected intraperitoneally with CdCl<sub>2</sub> (1.25 mg/kg/d) or PBS. After 5 wk, a cohort of each group received either HSP-1 T cells or PBS under the renal capsule as described in Materials and Methods. Tissue sections were graded by a blinded observer for parameters as described in Materials and Methods. Results were analyzed by nonparametric statistics using the Kruskal-Wallis one-way analysis of variance by ranks and the Mann-Whitney U test. \*  $p < 0.01$ ,  $^{\dagger} p < 0.005$ ,  $^{\S} p < 0.005$ .

cells in the infiltrates, and parameters of tubular cell damage for the four different experimental groups.

There are significant differences in each of these parameters between CdCl<sub>2</sub>-treated mice receiving HSP-1 T cells and all other subgroups. Some animals in the other groups demonstrated smaller, less severe infiltrates. Such infiltrates in the CdCl<sub>2</sub>-treated mice receiving subcapsular PBS, and in the PBS-treated group receiving HSP-1 cells, may represent disease related to local induction of HSP70 by surgical manipu-

lation. No infiltrates were observed in the contralateral, unmanipulated kidneys of any animals.

**HSP70-reactive T Cells Can Be Isolated from Nephritic CdCl<sub>2</sub>-treated Kidneys.** Our studies to this point demonstrated that T cells specific for an immunodominant HSP peptide could injure renal cells which had been induced to express HSP70 by CdCl<sub>2</sub> treatment either in vivo or in vitro. An important corollary to these studies was to examine whether, in fact, T cells infiltrating the nephritic kidneys of



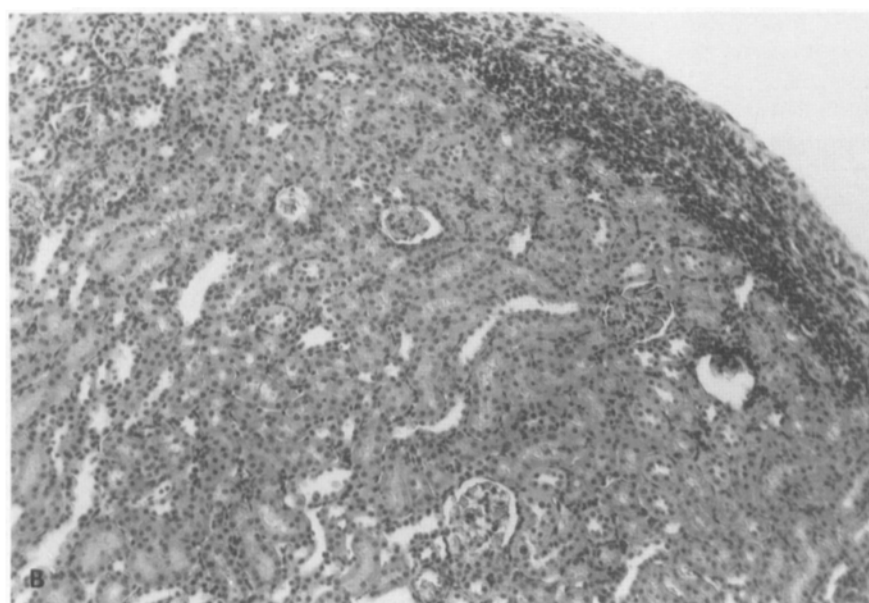
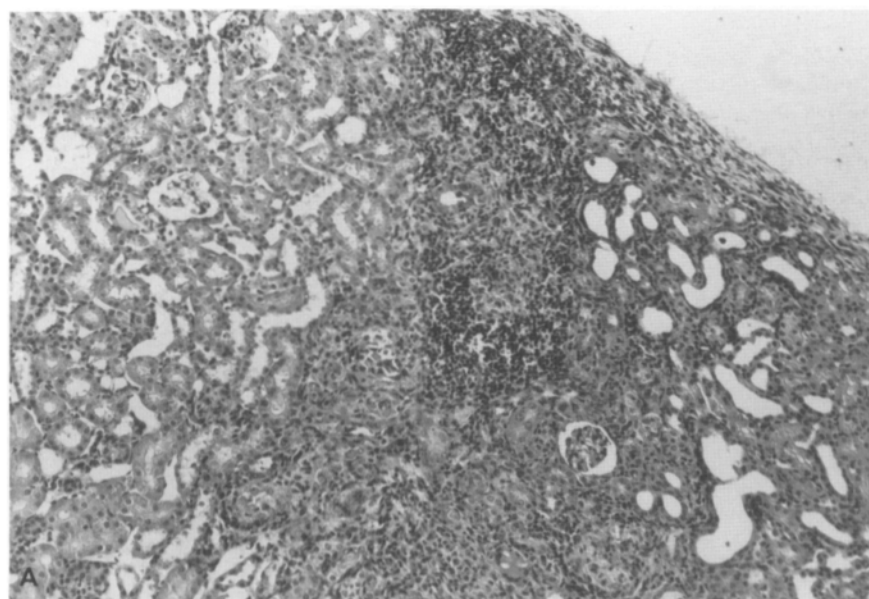
**Figure 6.** CdCl<sub>2</sub> T cells (HSP70-reactive, eluted from nephritic kidneys), but not HEL-reactive T cells are cytotoxic to heat-shocked or cadmium-treated tubular cells. MCT cells were either heat-shocked, treated with CdCl<sub>2</sub> for varying intervals or unmanipulated and then incubated with either CdCl<sub>2</sub> or HEL T cells at 37°C for 20 h. Percent cytotoxicity determined by metabolism of MTT as described in the Materials and Methods section. Each data bar represents the mean of triplicate samples of a representative experiment. (A) CdCl<sub>2</sub> T cells with heat-shocked and control MCT cells, (B) CdCl<sub>2</sub> T cells with cadmium-treated and control MCT cells; (C) HEL T cells with heat-shocked and control MCT cells; (D) HEL T cells with cadmium-treated and control MCT cells.  $p < 0.01$ , by analysis of variance and two-tailed T test.



**Table 2.** *Passive Transfer of Disease by HSP70-reactive CdCl<sub>2</sub> T Cells*

Pretreatment/cells	Infiltrate area	Inflammatory cells	Tubular damage
CdCl <sub>2</sub> /CdCl <sub>2</sub> (n = 9)	1.85 mm <sup>2</sup> ± 0.8*	2.6 ± 0.2 <sup>‡</sup>	3.67 ± 0.2 <sup>§</sup>
CdCl <sub>2</sub> /HEL (n = 10)	0.003 mm <sup>2</sup> ± 0.003	0.25 ± 0.2	0.4 ± 0.5
PBS/CdCl <sub>2</sub> (n = 9)	0.029 mm <sup>2</sup> ± 0.03	0.3 ± 0.3	0.4 ± 0.5
PBS/HEL (n = 9)	No infiltrates		

Animals were injected intraperitoneally with CdCl<sub>2</sub> (1.25 mg/kg/d) or PBS for 5 wk, after which time a cohort of each treatment group received either HEL or CdCl<sub>2</sub> T cells or PBS under the renal capsule. Tissue sections were graded by a blinded observer for parameters as noted in Materials and Methods. Results were analyzed by nonparametric statistics using the Kruskal Wallis one-way analysis of variance by ranks and the Mann Whitney U test for statistical significance. \*  $p < 0.05$ , <sup>‡</sup>  $p < 0.01$ , <sup>§</sup>  $p < 0.01$ .



**Figure 7.** CdCl<sub>2</sub> T cells, but not HEL T cells induce renal parenchymal infiltrates on passive transfer. SJL/J mice chronically treated with CdCl<sub>2</sub> were injected under the renal capsule with 0.075 ml of T cell suspension at  $2.5 \times 10^6$  cells/ml, and the kidneys harvested after 1 wk, fixed, sectioned, and stained with hematoxylin and eosin. Representative hematoxylin- and eosin-stained sections show typical parenchymal infiltrates observed after transfer of CdCl<sub>2</sub> T cells. In contrast, despite the presence of HEL T cells under the renal capsule following transfer, no parenchymal infiltration is observed. (A) CdCl<sub>2</sub> T cell transfer; (B) HEL T cell transfer.

mice treated chronically with CdCl<sub>2</sub> recognize HSP70. To investigate this possibility, we isolated T cells from the tubular fraction of diseased kidneys and cultured with them with the HSP 180-196 peptide. These T cells were derived from mice treated for 13 wk with CdCl<sub>2</sub> (1.25 mg/kg/d) since the interstitial infiltrates predominate at this time point. We also developed an HEL-reactive T cell line as a control cell line which would not be expected to recognize any renal antigen.

The antigen specificity of these two cell lines was determined by DTH response testing, as with the HSP-1 cell line. As illustrated in Fig. 4, B and C, each of these cell lines demonstrated a positive response to the appropriate antigen, but not to the irrelevant peptide. These studies showed that there are T cells infiltrating the kidneys of mice chronically treated with CdCl<sub>2</sub> that recognize the HSP 180-196 peptide. This kidney-derived cell line was designated CdCl. Both the CdCl and the HEL T cell lines, like HSP1, were CD4<sup>+</sup>, CD8<sup>-</sup>, and expressed TCR- $\alpha/\beta$ .

**CdCl<sub>2</sub> Cells Are Cytotoxic to Heat-shocked or Cadmium-treated Tubular Cells.** We next examined whether the CdCl<sub>2</sub> cell line was cytotoxic to stressed renal tubular cells. Fig. 6, A and B demonstrate that CdCl<sub>2</sub> cells mediate cytotoxicity against both heat shocked and CdCl<sub>2</sub>-treated MCT cells. Similar to the HSP1 cells, the degree of cytotoxicity is greater at any E/T ratio when the MCT cells have been exposed to CdCl<sub>2</sub> for longer periods of time. The HEL-specific cells did not mediate significant cytotoxicity to MCT cells at any E/T ratio, either under conditions of heat shock or CdCl<sub>2</sub> treatment (Fig. 6, C and D).

**CdCl<sub>2</sub> T Cells Mediate an Inflammatory Interstitial Nephritis after Adoptive Transfer.** The most stringent test of the pathogenicity of the CdCl<sub>2</sub> T cells is to examine whether they are capable of initiating an inflammatory interstitial nephritis after adoptive transfer to an appropriate host. As in the above-described studies with the HSP1 cells, we performed the cell line transfers to CdCl<sub>2</sub>-treated syngeneic mice at a time when tubular HSP70 was expressed (5 wk), but before the development of interstitial infiltrates. The HEL cells were additionally transferred as a control cell line, and both cell lines were transferred into PBS- as well as CdCl<sub>2</sub>-pretreated SJL recipients. As shown in Table 2, interstitial infiltrates were seen reproducibly with transfer of the CdCl<sub>2</sub> T cells into mice pretreated with CdCl<sub>2</sub>. In contrast, HEL T cells, although visible under the renal capsule after transfer, did not invade the renal parenchyma regardless of whether the animals had received chronic CdCl<sub>2</sub> injections. Representative sections from kidneys of CdCl<sub>2</sub>-treated mice that received subcapsular transfer of CdCl<sub>2</sub> or HEL T cells are shown in Fig. 7. As shown, CdCl<sub>2</sub> T cells (Fig. 7 A) invade the renal parenchyma after subcapsular transfer compared with HEL T cells that remain just under the renal capsule (Fig. 7 B).

## Discussion

The work presented in this paper is the first demonstration that the immune response to a neoantigen induced by "nonimmunologic" injury to the kidney can markedly am-

plify interstitial disease. We have shown that T cells specific for an immunodominant peptide of HSP70 are cytotoxic to tubular epithelial cells induced to express HSP70 by either heat shock or CdCl<sub>2</sub>. These T cells also mediate an inflammatory interstitial lesion after adoptive transfer into hosts induced to express HSP70 in tubular epithelium. We have also demonstrated that T cells infiltrating the kidneys of animals chronically receiving CdCl<sub>2</sub> injections recognize this immunodominant HSP70 peptide, are cytotoxic to stressed tubular cells, and can passively transfer histologic disease to HSP70-expressing recipients. HSP70 expression precedes and is necessary for inflammatory injury mediated by HSP70-reactive T cells in this model of toxin-induced interstitial nephritis. Although heavy metals, including cadmium, are toxic to tubular cells and might induce expression of mediators that could nonspecifically promote T cell accumulation in the renal parenchyma, our use of the HEL-reactive T cell line strongly argues against this interpretation. At the light microscopic level, the tubulointerstitial architecture was intact at the time of the cell transfers. Our studies demonstrating HSP70 reactive T cells in the kidneys of mice chronically treated with CdCl<sub>2</sub> do not, of course, rule out the presence of T cells with other specificities. We focused on HSP70 because it is immunogenic, easily inducible, and attractive as a potential neoantigen participating in renal injury.

A variety of toxins and stresses in intact kidneys and cultured renal cells induce HSP70 expression (8, 26, 27). Exposure to toxins such as cadmium and lead, which cause tubular dysfunction and chronic interstitial nephritis in exposed humans and rodents (24, 28–32), is typically chronic and sustained. Such circumstances could provoke prolonged HSP70 expression, and allow autoreactive, HSP70-specific T cells to expand in number and mediate injury. Heavy metal exposure is associated with differential activation of distinct T cell subsets, consistent with the Th1/Th2 paradigm. Mouse strains that develop polyclonal B cell activation after challenge with mercuric chloride display evidence for Th2 cell activation (33). In contrast, Th1-type cells mediating DTH responses inhibit tissue injury in rats with mercury-induced autoimmunity (34). In CdCl<sub>2</sub>-induced interstitial nephritis, preliminary studies suggest that activation of Th1 cells is critical for the resultant immunopathology. This is consistent with previous studies in other experimental models of interstitial nephritis that have confirmed a pathogenic role for renal tubular antigen reactive T cells which mediate DTH (23, 25, 35).

Our studies raise important second order questions regarding the requirements for CD4<sup>+</sup> HSP-reactive T cell injury to tubular epithelial cells. Recent studies suggest that T cell-mediated injury directed against HSPs may involve mechanisms distinct from conventional MHC-restricted T cell recognition of antigen-presenting cells. Heat shock or transfection with a constitutively expressed HSP65 gene increases T cell-mediated injury directed against tumor cells (36, 37). HSP70 recognition by some CD4<sup>+</sup> clones is independent of class II MHC or engagement of CD4 because HSP70 recognition may involve binding an HSP-specific cell surface receptor. This possibility is supported by the recent

finding that an HSP70 participating in antigen presentation is detectable on the cell surface (38).

Our model system supports the notion that at least some of the recognition of tubular epithelial cells involves the antigen receptor, since the HEL-specific T cells clearly do not mediate the same effect as either of the two HSP-reactive cell lines. Recognition of HSP70 by these CD4<sup>+</sup> T cells is presumably class II restricted. Previous studies have shown that murine macrophages transfected with HSP65 present these peptides with class II MHC (39). Other examples of endogenous peptides presented in conjunction with class II MHC molecules involve antigens that access the secretory pathway (40, 41) or cytosolic antigen (42). Some HSPs may be secreted into the extracellular space (43) and cytosolic antigen access to the lysosomal compartment may involve HSPs (44). Since HSPs are associated with nascent class II and invariant chain during antigen processing and with trafficking of class II to the cell membrane (38), failure of appropriate dissociation from class II MHC in the endosomal compartment might allow aberrant presentation of HSP70 in the context of class II.

Several pieces of evidence suggest that HSP70-reactive T cells may comprise a subset of immunocompetent cells primed for self-reactivity (11, 45, 46). They may also have a role in

disruption of normal peripheral mechanisms of tolerance. Stimulation of peripheral blood mononuclear cells with purified protein derivative and HSP65 induces both antigen-specific and nonspecific cytotoxic activity by CD4<sup>+</sup> MHC class II-restricted T cells (47). Self-reactive T cells from neonatally thymectomized BALB/c mice show enhanced proliferation after culture with HSP-65 compared with non-self-reactive T cells (48), and T cell subsets from nude mice responsive to HSP65 secrete significant levels of IL-2, which abrogates the development of clonal anergy (49). These findings suggest that after induction of endogenous HSPs, populations of HSP-specific autoreactive cells may not only be resistant to peripheral tolerogenic mechanisms, but also interfere with normal regulation of non-HSP-reactive autoimmune T cell clones. Such dysregulation might permit the emergence of autoimmune disease.

In conclusion, our experiments indicate that HSP-reactive T cells are capable of mediating tubulointerstitial damage in response to a neoantigen induced by a renal tubular cell toxin, CdCl<sub>2</sub>. These studies raise the intriguing possibility that immune responses to stress induced antigens play an important pathogenic role even in renal diseases not currently classified as immune mediated.

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